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CsHPt1, a putative histidine-containing phosphotransmitter protein induced during early somatic embryogenesis in Valencia sweet orange

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Abstract

A cDNA named CsHPt1 (for *Citrus sinensis* histidine phosphotransmitter protein 1) containing a conserved domain characteristic of a two-component phosphorelay intermediate associated with signal transduction mechanisms, was isolated from globular embryos of *C. sinensis* sweet orange cv. 'Valencia' by differential display RT-PCR. The corresponding full-length cDNA, subsequently obtained by RACE-PCR, is 772 bp with a 450 bp open reading frame encoding a 150 amino acid protein. The deduced amino acid sequence of CsHPt1 is approximately 60% similar to five AHP histidine-containing phosphotransmitter (HPt) proteins from *Arabidopsis thaliana* and contains the 12 amino acid motif characteristic of all known HPt domains.

RT-PCR analyses showed higher CsHPt1 transcript abundance in embryogenic callus when compared to non-embryogenic callus, leaves, phloem, roots, and flavedo (rind). Immature fruits and mature flowers, both of which contain tissues with embryogenic potential, also showed increased CsHPt1 expression. Furthermore, CsHPt1 gene expression levels in globular embryos were higher than in embryogenic callus in three of the five cell lines we analyzed. Results of this study provide insight on signal transduction mechanisms that may be active during early plant embryogenesis.
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Keywords: *Citrus sinensis*; Somatic embryogenesis; Differential display; Histidine-containing phosphotransmitter protein; Signal transduction

1. Introduction

Somatic embryogenesis is an attractive system to study changes in gene expression during plant embryogenesis. Somatic embryos go through the same stages of embryogenesis as their zygotic counterparts, and there is extensive evidence that both forms of embryogenesis share common molecular mechanisms. In addition, somatic embryogenesis has the advantage of allowing experimental manipulation of culturing conditions, providing easy access to embryos at different developmental stages [1–4]. In recent years, much effort has been directed to the identification of genes with altered expression patterns during somatic embryogenesis [5,6]. Although several embryogenesis-related genes have been identified in carrot and other species [7–9], there are only a few reports on genes induced early in embryogenesis, particularly those regulating the transition from somatic to embryogenic cells [10,11]. Early embryogenesis genes reported thus far include the somatic embryogenesis receptor kinase-1 gene, SERK, first isolated in carrot [12] and later in Arabidopsis [13], that marks the transition of somatic into embryogenic cells; the WUSCHEL gene in Arabidopsis coding for a homeodomain protein that promotes the vegetative-to-embryogenic transition [14] and a transcription factor encoded by the LEAFY COTYLEDON2 (LEC2) gene, which regulates early embryo development in Arabidopsis [15].

Citrus is a facultative apomict that reproduces by adventive embryony of the nucellus. Callus derived from

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the nucellus retains the high embryogenic potential of this maternal tissue [16–18]. Somatic embryogenesis in callus can be induced when sucrose is replaced by alternate carbon sources such as glycerol [19], galactose [20,21], lactose [22], or maltose [23,24]. Vu et al. [25] concluded that sweet orange (*Citrus sinensis* L. Osbeck) embryogenic cells can efficiently utilize glycerol as a carbon source as evidenced by high activities of sucrose phosphate synthase, sucrose synthase, invertase, and the accumulation of sugar and starch in callus grown in glycerol-supplemented media. Somatic embryos in sweet orange can also be induced when embryogenic callus is cultured onto semi-permeable membranes of cellulose acetate [26]. In an attempt to identify genes expressed during early somatic embryogenesis in *C. sinensis*, we used differential display RT-PCR to compare gene expression patterns between embryogenic callus at the pre-globular embryo stage and embryogenic callus containing globular-stage embryos.

2. Materials and methods

2.1. Embryogenic callus

Embryogenic callus lines (V89, V97, V98, V99, and V00) of *C. sinensis* cv. 'Valencia' were initiated (e.g., V89 initiated in 1989) and maintained as described by Kobayashi et al. [27]. Callus was subcultured every 21 days, grown under low light (15–20 $\mu E\ m^{-2}\ s^{-1}$) provided by cool-white fluorescent lamps, constant 27 °C, and a 4 h photoperiod.

2.2. Non-embryogenic callus

A non-embryogenic cell line was developed from epicotyl explants of 3-week germinated nucellar seedings of *C. sinensis* cv. 'Valencia'. Seeds were surface sterilized and cultured on MT basal medium [28] and cultured in the dark at 27 °C for three weeks. Epicotyl explants were cultured on MT basal medium supplemented with 1 μM 6-benzylaminopurine (BA; Sigma, St. Louis, MO), 2.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma), and 100 mg/l casein hydrolysate to induce callus. The epicotyl cultures were subcultured every 21 days and grown in a growth cabinet under low light (15–20 $\mu E\ m^{-2}\ s^{-1}$), provided by cool-white fluorescent lamps, constant 27 °C, and a 4 h photoperiod. After 12 months of selection, a rapidly growing slightly friable callus was obtained. The established callus line was maintained in polycarbonate GA-7 vessels with a polypropylene closure (Magenta Corp., Chicago, IL) using the same culture medium but with 1 μM 2,4-D and a 28-day subculture period.

2.3. Embryo induction

To induce globular-stage embryos, embryogenic callus was subcultured onto MT medium where the sucrose was replaced by 2% (w/v) glycerol as the primary carbon source, and grown for 21–30 days.

2.4. RNA isolation

Total RNA was isolated from embryogenic callus at the pre-globular embryo stage, embryogenic callus containing globular-stage embryos (35 days after induction), non-embryogenic callus and from several tissues of *C. sinensis* cv. 'Valencia' using the RNeasy™ Plant mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. For calli, material from three independent culture plates was pooled before RNA isolation. RNA extracts were treated with DNase I (Amersham Pharmacia, Piscataway, NJ) to remove contaminating DNA. RNA integrity was confirmed by electrophoresis in agarose gels with ethidium bromide staining.

2.5. RNA differential display

Differential display reactions were performed following the method of Liang and Pardee [29]. DNase-treated total RNA from embryogenic pre-globular-stage callus and globular-embryo stage callus (35 days after induction) were subjected to RT-PCR using the RNA Image Kit 1 in conjunction with four primer sets (GenHunter®, Nashville, TN) following the manufacturer's recommendations. Partial cDNAs from bands, showing differential intensity after electrophoresis on 6% polyacrylamide gels, were isolated and reamplified. The resulting PCR products were purified using the QIAquick™ PCR purification kit (Qiagen) and cloned into the pCR2.1 TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA).

2.6. DNA sequencing and homology searches

Nucleotide sequencing was performed using the ABI Prism® BigDye™ Terminator Cycle Sequencing kit and the ABI PRISM 3700 automated sequencer (Applied Biosystems, Foster city, CA). Sequence analysis was carried out using Sequencher® v 4.2 software (Gene Codes Corp., Ann Harbor, MI) with BLAST tools [30] used for DNA homology searches of the GenBank® database. The Conserved Domain Architecture Retrieval Tool (CDART) from NCBI was used for conserved protein domain searches [31].

2.7. Primer design

Primers were designed based on cDNA sequences obtained by differential display using Primer3 software [32]. Specific primers, 5′-TCTGCCATTGATGACAAGGA-3′ and 5′-AGCCAGCTCTCTATGCTTGG-3′ were used to generate a 160 bp amplicon for CsHPt1. A partial sequence of the 18S ribosomal RNA gene from *Citrus aurantium* L. was used to design specific primers 5′-GCTTAGGCCAAG-

GAAGTTTG-3′ and 5′-TCTATCCCCATCACGATGAA-3′. These produced an amplicon of 152 bp and served to normalize the RT-PCR expression data for CsHPt1. All primers were tested for specificity by PCR, followed by electrophoresis. Generation of a single PCR band on a gel, when using sweet orange cDNA as a template, was followed by cloning and DNA sequencing to confirm identity of the PCR products.

2.8. 5′ RACE to obtain full-length CsHPt1 cDNA

The FirstChoice™ RLM-RACE kit (Ambion, Austin, TX) was used to generate the 5′ sequence upstream of CsHpt1 cDNA. Ten micrograms of embryogenic calli total RNA was treated with calf intestinal phosphatase and then with tobacco acid pyrophosphatase. A 5′ RNA adapter was ligated to the treated RNA and the resulting products reverse transcribed using an oligo $(dT)_{14}$ primer at 42 °C for 60 min. Outer primers specific for the RLM-RACE adapter (5′-GCTGATGGCGATGAATGAACACTG-3′) and CsHPt1 (5′-CCCAAGATTGAGAATGTGTGCAAG-3′) were used in a primary PCR reaction consisting of 1× PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton® X-100) 1.5 mM $MgCl_2$, 200 uM each of dCTP, dATP, dTTP, and dGTP, 10 pmol of forward and reverse primer, and 2.5 U of Taq DNA polymerase (Promega, Madison, WI) in a 25 μl reaction volume. Amplification was performed using a PTC-200 thermal cycler (Bio-Rad, Hercules, CA) under the following conditions: 95 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 5 min. PCR products (1 μl) were used as template in a nested reaction utilizing the RACE inner primer 5′-CGCGGATCCGAACACTGCGTTTGCT-GGCTTTGATG-3′) and an inner gene specific primer (5′-GTTCAACTCTTGCTGAAGGCTGGT-3′) designed from the CsHPt1 cDNA. The resulting products were electrophoresed at 100 V through a 2% TAE agarose gel stained with ethidium bromide. Candidate bands were excised and purified using a QIAquick® Gel Extraction kit (Qiagen), subsequently cloned into the pPCR2.1 TOPO vector using the TOPO TA Cloning Kit (Invitrogen), and sequenced.

2.9. RT-PCR analysis

RT-PCR analysis was performed using the TITAN™ one-tube RT-PCR kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's instructions. DNase-treated RNA (50 ng) was used as a template in RT-PCR with CsHPt1 primers for 35 cycles and with 18S primers for 15 cycles to ensure that the amplification reaction was in the linear range. RT-PCR products were visualized after agarose gel electrophoresis and ethidium bromide staining. Images were captured using the Kodak™ 1D Scientific Imaging System (Eastman Kodak Co., New Haven, CT).

Real-time RT-PCR was performed with the iCycler® (Bio-Rad) and the QuantiTect™ SYBR® Green RT-PCR kit (Qiagen). Ten-fold serial dilutions were prepared for each DNase-treated RNA sample using the BioMek® 2000 automated robot (Beckman Coulter, Fullerton, CA) and for generation of standard curves. Reactions were carried out in a total volume of 25 μl using the following temperature profile: 50 °C for 30 min for the RT step, 95 °C for 15 min for the hot-start polymerase activation step, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 60 s and a final extension step of 72 °C for 5 min. PCR amplification was followed by DNA melting curve analysis to verify primer specificity, identity of the RT-PCR products and absence of primer-dimer formation. PCR amplification efficiencies for each sample were determined from their corresponding standard curves. Statistical analysis (*t*-test) was performed using Microsoft Excel® (Microsoft, Redmond, WA) and relative expression levels were determined using the comparative method for real-time RT-PCR as described by Livak and Schmittgen [33].

2.10. Southern blot analysis

Restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Xho*I, *Cla*I, and *Xba*I were used to digest genomic DNA isolated from *C. sinensis* cv. Valencia. The resulting DNA fragments were separated by electrophoresis in a 0.8% TAE agarose gel and subsequently transferred to a Hybond N+ positively charged nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by capillary blotting [34]. A radiolabeled probe was generated using the Prime-a-Gene® Labeling System (Promega) in the presence of $[\alpha^{33}P]$-dCTP (3000 ci/mmol, MP Biomedicals, Irvine, CA). Unincorporated nucleotides were removed by passing the reactions through a ProbeQuant™ G-50 column (Amersham Pharmacia Biotech). Both prehybridization and hybridization were performed at 65 °C in the following buffer: 6× SSPE, 7× Denhardt's solution (Amresco, Solon, OH), 1% SDS, and 100 μg/ml denatured salmon sperm DNA. Washing was performed at high stringency under the following conditions: 5× SSC, 0.5% SDS at room temperature followed by 1× SSC, 1% SDS at 37 °C with a final wash in 0.1× SSC, 1.0% SDS at 65 °C. Detection and image acquisition were performed using a Cyclone® phosphor storage system (Perkin-Elmer, Boston, MA).

3. Results

3.1. Somatic embryogenesis in C. sinensis

Embryogenic callus of sweet orange is white and friable, has a grainy texture and a pleasant aromatic odor [35] (Fig. 1A). When embryogenic callus was cultured on glycerol-based media, globular-stage embryos developed after 21–30 days (Fig. 1B). Sweet orange non-embryogenic callus (Fig. 1C) is off-white, less friable, and has a smoother texture than embryogenic callus. In addition, non-embryogenic callus

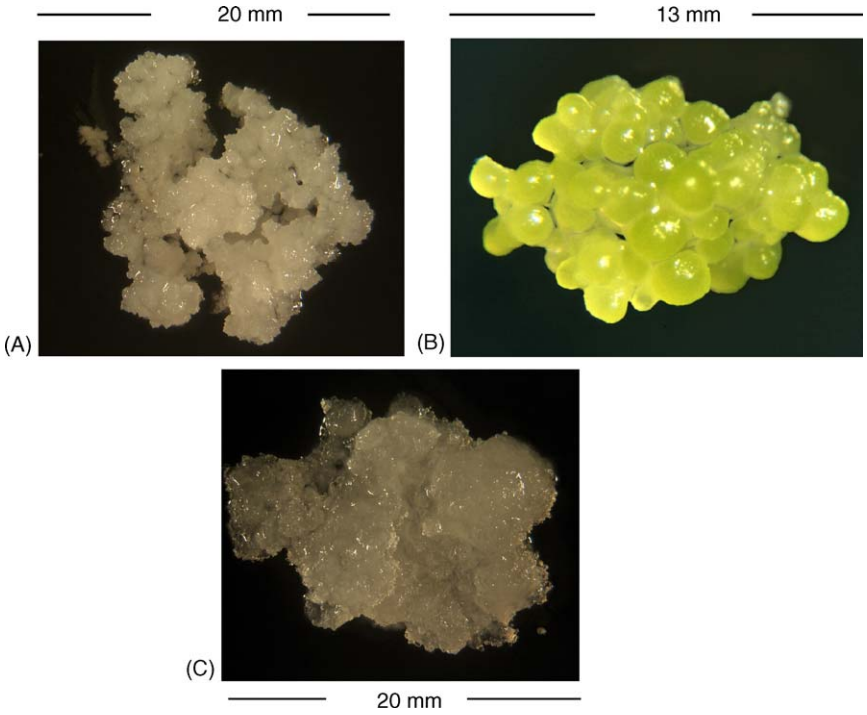

Fig. 1. Embryogenic and non-embryogenic calli of Valencia sweet orange line. (A) Embryogenic callus, (B) cluster of globular embryos emerging from embryogenic callus, 42 days after glycerol induction, and (C) non-embryogenic callus.

does not form embryos when cultured on glycerol medium (Niedz, personal communication). Five embryogenic cell lines were used in this study: V89, V97, V98, V99, and V00; all were morphologically similar.

3.2. Differential display and sequence analysis of CsHPt1

In an effort to isolate early embryogenesis genes in sweet orange, we used the differential display method to compare patterns of gene expression in embryogenic calli at the pre-globular embryo stage with embryogenic callus containing globular-stage embryos (35 days after induction). Approximately 40 cDNA bands showed differential signal intensity between both samples (data not shown). Six cDNA bands corresponding to genes with enhanced expression in globular-stage embryos were isolated from the polyacrylamide gels, cloned and sequenced. GenBank® database searches revealed that four of the cDNAs displayed homology to an aconitase-iron regulated protein in *Citrus limon* (L.) Burm. f. (AAC26045.1), a polyubiquitin precursor in developing fruits of *C. sinensis* (CK936407), a putative zinc-binding protein in immature ovaries of *Poncirus trifoliate* L. (NP194504.1) and an anthranilate phosphoribosyl transferase-like protein in Arabidopsis (AAK53020.1).

BLASTN analysis of a 452 bp cDNA, named CsHPt1 (for *C. sinensis* histidine phosphotransmitter protein 1), showed 99% sequence identity to a 799 bp cDNA clone from the peel of mature *C. sinensis* cv. 'Washington Navel' fruit (CB610784) whose sequence matches a putative histidine-containing phosphotransmitter protein. The 5′ RACE approach to obtain the full-length mRNA sequence of CsHPt1 resulted in a 772 bp fragment containing a 450 bp open reading frame (Fig. 2A). A conserved domain search of the 150 deduced amino acid sequence of CsHPt1 showed a region of 135 residues with 90.7% sequence alignment to the KOG4747 conserved domain, a two-component phosphorelay intermediate involved in MAP kinase regulation acting in signal transduction mechanisms (Fig. 2B). The CsHPT1 sequence contains the 12 amino acid motif identified as highly conserved in HPt domains, which begins one amino acid upstream from the histidine phosphorylation site [36]. Amino acid sequence alignment of CsHPt1 and five different members of the Arabidopsis His-to-Asp phosphorelay family showed approximately 60% homology. Furthermore, a 32 amino-acid sequence that has been identified as a signature sequence for HPt domains [37], is common to all Arabidopsis HPt family members, as well as CsHPt1 (Fig. 3).

3.3. Expression of CsHPt1 is enhanced during embryogenesis

To determine if CsHPt1 transcript abundance is increased in cell line V99 during somatic embryogenesis, we compared non-embryogenic calli to embryogenic calli at the pre-globular embryo stage by RT-PCR analysis. Three independent cultures were pooled for each callus type to

(A)

```
GGATCACCGATAATTTCTCTTTGAAACAAGAAAAAGACAATGGTGGGTAC
                                          M  V  G  T
CAGCCTTCAGCAAGAGTTGAACAACTTTGTTCGCTCCCTGCGTGAGCAGG
 S  L  Q  Q  E  L  N  N  F  V  R  S  L  R  E  Q  G
GTATACTGGACCATAATTTCGATACCTTGAGCAGAATTCAAAACGATCAG
  I  L  D  H  N  F  D  T  L  S  R  I  Q  N  D  Q
AGTCCTCTGTTTGTTACAGAAGTGATCAATTTGTTCACAAGGGATGCTGA
S  P  L  F  V  T  E  V  I  N  L  F  T  R  D  A  E
AAATGCCATAACACAAGCTCGTGATTCCCTGCAAGAACCTTCAGTGGACT
 N  A  I  T  Q  A  R  D  S  L  Q  E  P  S  V  D  Y
ATGACAAGTTGATAGCTGCGGTCCATCAACTCAGGGGAGCCAGCTCGAGC
  D  K  L  I  A  A  V  H  Q  L  R  G  A  S  S  S
ATTGGTGGTTGTCGCGTGGCCCTTGATTGCCGTGAACTTCGCTCTGCCAT
I  G  G  C  R  V  A  L  D  C  R  E  L  R  S  A  I
TGATGACAAGGACAAGGAAAGGTGTAATGAAATTCTCCAAAGGATTGTGG
 D  D  K  D  K  E  R  C  N  E  I  L  Q  R  I  V  E
AGGAGTACCAAACTTTGCATGTGAATCTTGCACACATTCTCAATCTTGGG
  E  Y  Q  T  L  H  V  N  L  A  H  I  L  N  L  G
AGGCAAATCCTGGCACTTCAGAGGGGACAACACCAAGCATAGAGAGCTGG
R  Q  I  L  A  L  Q  R  G  Q  H  Q  A
CTTGGTGTCTTTGCTGCTGGTGGTTCCTCAGCCGAACATCTAAATGGCTG
CTTATCATATAGCTTGTGTTAAATAAAGTTCACCAGAAACTAAACTTGGT
GCTCGTTTGAAACTATTTCGAGTTGGTGTCCTGTTTTATGTTGTGTTTGA
ACATCCGTGTTGCTTTCTTGTTTGTTGTTTTTGAACCTAATAACCTGTTT
TATGTTGTGTTTGAACACCAGTGTCGCTTCTTATTTGCTGTTTTTGAACT
TAATGAATAATTGTTGTTTGTTAAAAAAAAAAAAAAAAAAAAAAAAAA
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(B)

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CsHpt1          MVGTSLQQELNNFVRSLREQGILDHNFDTLSRIQNDQSPLFVTEVINLFTRDAENAITQA
KOG4747         ----SMQRDVSDYTKSLFDQGILDSQFLQLQELQDDSSPDFVEEVVGLFFEDSERLINNL
                    : *:::.:::.:** :***** :*  *..:*:*.** ** **:.** .*:*. *.:
CsHpt1          RDSLQEPSVDYDKLIAAVHQLRGASSSIGGCRVALDCRELRSAIDDKDKERCNEILQRIV
KOG4747         RLALD-CERDFKKLGSHVHQLKGSSSSIGALKVKKVCVGFNEFCEAGNIEGCVRCLQQVK
                * :*:  . *:.** : ****:*:*****. :*   *  :..  :  : * * . **::
CsHpt1          EEYQTLHVNLAHILNLGRQILALQRGQHQA
KOG4747         IEYSLLKKKLETLFQLERQEI---------
                 **. *: :*  :::* ** :
```

Fig. 2. (A) The full-length cDNA and deduced amino acid sequence of the CsHPt1 gene. The phosphotransfer domain region containing the histidine phosporylation site (in bold) is underlined. Amino acid symbols are written below the first nucleotide of the corresponding codon. (B) Alignment of the deduced amino acid sequence of CsHPt1 and the conserved domain KOG4747, a two-component phosphorelay intermediate involved in MAP kinase cascades. The boxed residues contain the 12-amino acid highly conserved motif of histidine phosphotransfer (HPt) domains, which contain the invariant histidine.

increase the significance of the analysis. RT-PCR analysis after 35 amplification cycles showed that CsHpt1 transcript abundance was higher in embryogenic calli when compared to non-embryogenic calli (Fig. 4).

Using RT-PCR analysis, we compared expression levels of CsHPt1 in different sweet orange tissues, specifically, leaves, flowers, phloem, immature fruits, roots, and flavedo (rind), together with those from non-embryogenic and pre-

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AHP2            NMARALDTTGTVDFSQVGASVHQLKGSSSSVGAKRVKTLCVSFKECCEAKNYEGCVRCLQ
AHP3            NMARALDQTGNVDFKLVGSSVHQLKGSSSSVGAKRVKGLCVTLKECCDSQNYEGCVRCLQ
AHP5            TMSISLERPDNVDFKQVDSGVHQLKGSSSSVGARRVKNVCISFKECCDVQNREGCLRCLQ
AHP1            DLSLSLDQQ-VVDFKKVDPHVHQLKGSSSSIGAQRVKNACVVFRSFCEQQNVEACHRCLQ
AHP4            NIDQALERG-SFDFNRLDSYMHQFKGSSTSIGASKVKAECTTFREYCRAGNAEGCLRTFQ
CsHPt1          QARDSLQEP-SVDYDKLIAAVHQLRGASSSIGGCRVALDCRELRSAIDDKDKERCNEILQ
                    :*:    .*:. : . :**::*:*:*:*. :*   *  ::.     : * * . :
```

Fig. 3. Sequence alignment of the deduced amino acid sequence of HPt domains from CsHPt1 and five histidine phosphotransfer proteins in *Arabidopsis thaliana* (AHP genes): AHP2 (NP_189581), AHP3 (BAA37111), AHP5 (NP_563684), AHP1 (NP_188788), and AHP4 (AB041766). Residues in bold form the 32 amino acid sequence that represent the specific signature for HPt modules: (EDNQ)X_3(LASIFV)X_4**H**(KRAHQGFTS)(LIF) (HKARGS)(GS)(GASMW)(-CADTFWGS)X_3(GDNRHE)X_3(IMSLVPAG)X_6(ILAFNS) (EQMLKRN), where alternative residues at one position are in brackets, X represents any residue and the histidine phosphorylation site is in bold [37]. The highly conserved consensus motif of Hpt domains is boxed.

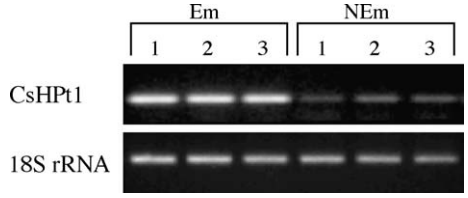

Fig. 4. RT-PCR analysis of total RNA from embryogenic (Em) and non-embryogenic (NEm) callus with CsHPt1 and 18S ribosomal RNA primers. After agarose gel electrophoresis, the RT-PCR products were visualized by ethidium bromide staining. Numbers represent RNA from calli of three replicate experiments.

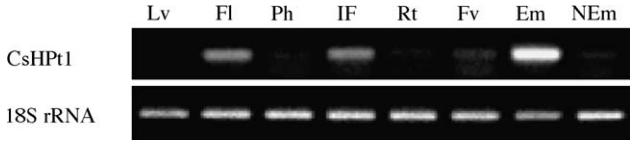

Fig. 5. RT-PCR analysis of CsHPt1 in sweet orange tissues using total RNA with CsHPt1 and 18S ribosomal RNA primers. After electrophoresis, RT-PCR products were visualized with ethidium bromide staining. Lv, leaves; Fl, flowers; Ph, phloem; IF, immature fruits; Rt, roots; Fv, flavedo; Em, embryogenic calli; NEm, non-embryogenic calli.

globular embryogenic callus from cell line V99 (Fig. 5). The highest CsHPt1 transcript level was found in embryogenic calli. Flowers and immature fruits, both of which contain potential embryogenic tissues, showed an enhanced CsHPt1 expression over the remainder of the tissues, though not higher than in embryogenic calli. In contrast, leaves, phloem, roots, flavedo, and non-embryogenic calli showed very low abundance of CsHPt1 transcripts.

To compare CsHPt1 transcript abundance between pre-globular-stage embryogenic calli and 35-day embryogenic calli containing globular embryos in cell line V99, we used real-time semiquantitative RT-PCR analysis. Specific primers were designed for both CsHPt1 and for the reference transcript (18S rRNA). A single melting temperature peak for each PCR product confirmed the specificity of the primers (data not shown). Cycle threshold (Ct) values for 18S rRNA were very similar in both pre-globular embryogenic callus (Ct = 10.78 ± 0.04, Table 1) and globular embryos (Ct = 10.45 ± 0.17) and were used to normalize the CsHPt1 data. We found that the difference in CsHPt1 transcript abundance between pre-globular embryogenic calli and globular embryos in cell line V99 was highly significant ($P < 0.01$), with globular embryos (Ct = 16.93 ± 0.19) containing approximately seven times more CsHPt1 transcripts than pre-globular embryogenic callus (Ct = 20.12 ± 0.10). To test if these results were similar for other embryogenic cell lines from Valencia sweet orange, we repeated the experiments using callus from cell lines V89, V97, V98, and V00. We found that CsHPt1 was expressed in all four additional cell lines; however, only lines V89 and V98 showed highly significant differences ($P < 0.01$) between the two stages, with globular embryos containing higher transcript levels than embryogenic calli. Lines V97 and V00 showed no significant difference in CsHPt1 transcript levels between the two stages.

Results of Southern analysis suggest that CsHPt1 is present as a single copy gene in the *C. sinensis* genome (Fig. 6). All restriction enzymes used in the DNA digestions generated a single DNA band after hybridization to a CsHPt1 specific probe, with the exception of *Eco*RI and *Cla*I, which produced three and two bands, respectively. The smaller bands generated by these two enzymes appear to arise from multiple digestion sites within a single and larger DNA sequence.

Table 1
Real-time quantitative RT-PCR analysis of CsHPt1 in embryogenic callus and callus containing globular embryos using the comparative method

Cell line	Sample	Ct value[a]		ΔCt	ΔΔCt[b]	Fold difference ($2^{-\Delta\Delta Ct}$)
		CsHPt1	18S			
V99	EC	20.12 ± 0.10	10.78 ± 0.04	9.34 ± 0.10	0	
	GE	16.93 ± 0.19	10.45 ± 0.17	6.48 ± 0.17	−2.86	7.3**
V89	EC	22.63 ± 0.12	10.60 ± 0.29	12.03 ± 0.23	0	
	GE	14.87 ± 0.12	10.60 ± 0.29	4.2 ± 0.27	−7.83	227.5**
V97	EC	17.23 ± 0.03	10.77 ± 0.14	6.46. ± 0.15	0	
	GE	17.33 ± 0.13	10.80 ± 0.01	6.53 ± 0.13	0.07	n.s.
V98	EC	20.93 ± 0.09	11.27 ± 0.12	9.66 ± 0.15	0	
	GE	13.73 ± 0.09	10.80 ± 0.06	2.93 ± 0.10	−6.73	106.1**
V00	EC	22.70 ± 0.25	11.63 ± .1.2	11.07 ± 1.22	0	
	GE	19.20 ± 0.75	11.77 ± .1.2	7.43 ± 1.4	−3.64	n.s.

n.s., not significant.

[a] Ct values are expressed as the mean and SE of three replicate experiments. Ct is the cycle number at which fluorescence exceeds a threshold value within the exponential range of the amplification reaction.

[b] Represents the difference in relative expression of CsHPt1 between embryogenic calli (EC) and calli containing globular embryos (GE).

** Significantly higher expression ($P < 0.01$) of CsHPt1 in EM when compared to GM based on a Student's *t*-test using three independent replications.

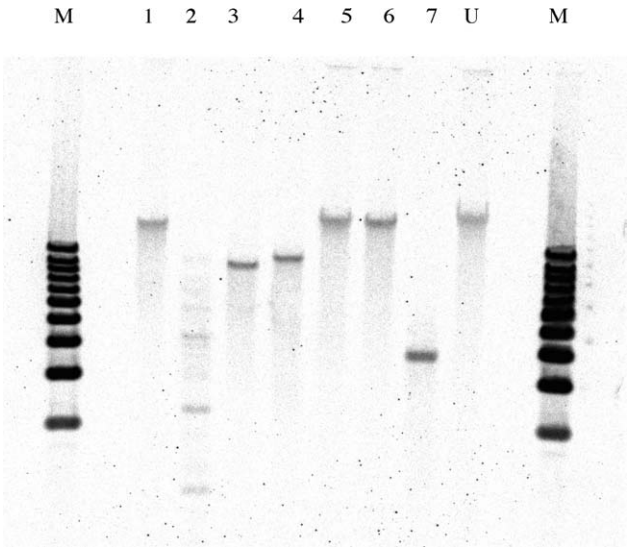

Fig. 6. Southern blot analysis of the CsHPt1 gene in *Citrus sinensis*. Genomic DNA was digested with several restriction enzymes and after agarose electrophoresis, blotted onto a positively charged nylon membrane. Hybridization to a [α^{33}P]-dCTP-probe generated with primers specific to CsHPt1 cDNA followed. Restriction enzymes used were: 1, *Bam*HI; 2, *Eco*RI; 3, *Hin*dIII; 4, *Pst*I; 5, *Xho*I; 6, *Cla*I; 7, *Xba*I. M, molecular markers; U, undigested genomic DNA.

4. Discussion

A variety of methods have been used to identify genes with altered expression patterns during somatic embryogenesis. Most of the genes found to be upregulated during this process are related to late embryogenic stages while only few reports focus on early embryogenesis genes [14]. We have isolated a gene named CsHPt1, encoding a histidine-containing phosphotransmitter protein that is expressed during early somatic embryogenesis in Valencia sweet orange and whose expression is also enhanced in other tissues with embryogenic potential. Although HPt intermediates of two-component signal transduction mechanisms have been identified previously in Arabidopsis [39] and other species [40,41], their role in early embryogenesis has not been reported.

Somatic embryogenesis shares cellular and molecular mechanisms with zygotic embryogenesis. Re-programming of gene expression in somatic cells occurs through a complex signaling system that induces and coordinates the expression of a particular set of genes inherent to the process of embryogenesis. A key area of plant development research has been to identify possible signaling factors that activate and regulate expression of genes with critical roles in early embryogenesis [38]. Transcription factors coded by LEC1, LEC2, and FUSCA have been shown to play significant roles during early plant embryogenesis in Arabidopsis [15]. Many other members of signaling pathways underpinning the embryogenic program, however, remain to be identified.

Using the differential display methodology, we have isolated and characterized a cDNA representing CsHPt1, whose expression is higher in embryogenic than in non-embryogenic calli (Fig. 4). Expression of CsHPt1 is also enhanced in flowers and immature fruits, two tissues with embryogenic potential (Fig. 5). The 150 bp deduced amino acid sequence of CsHPt1 contains the conserved functional domain KOG4747 (Fig. 2), characteristic of phosphotransmitter proteins that are part of two-component signal transduction systems found in numerous prokaryotic and eukaryotic organisms [42]. These systems consist of a histidine protein kinase, localized in the cell membrane that senses a signal input, and a response regulator that mediates the output. When the histidine protein kinase senses an environmental cue it initiates a signal transduction pathway by phosphorylating its own conserved histidine residue and transferring the phosphate to a conserved aspartate residue in the response regulator. Higher plants utilize two-component systems to mediate their response to biotic and abiotic stimuli such as hormones, stress and light signaling [44]. In some cases, the sensor protein kinase and the response regulator utilize histidine-to-aspartate phosphorelay intermediates comprised of single domain proteins, the histidine-containing phosphotransmitter (HPt) proteins [43]. Completion of the Arabidopsis genome sequence has revealed 54 genes encoding proteins that are part of two-component signaling systems [44]. Five of these genes, the AHP genes (AHP1 through AHP5), encode signal transducers comprised of a single HPt domain [45,46]. AHP2 and AHP3 are 81% identical to each other, while AHP2 and AHP1 share 45% identity [44]. Recent studies have determined that at least three of the AHP factors are involved in cytokinin transduction pathways [46,47]. Moreover, in the Arabidopsis His-to-Asp phosphorelay system several genes for cytokinin receptors, the histidine kinase (AHK) genes, have also been identified, as well as the response regulator (ARR) genes, that encode transcription factors. The AHP factors in Arabidopsis have been shown to be located mainly in the cytoplasm and have the ability of interacting with cytokinin receptors encoded by the AHK genes [43].

The deduced amino acid sequence of CsHPt1 is approximately 60% similar to the members of the AHP family in Arabidopsis. This limited degree of homology is not surprising. Except for a 12 amino acid motif containing the invariant phosphorylated histidine, amino acid sequence among HPt domains are poorly conserved and are more likely to share similar fold and common features related to the mechanism for histidine–aspartate phosphoryl transfer [48]. After extensive analysis of the alignment of all known HPt amino acid sequences, Rodrigue et al. [37] identified a 32 amino acid sequence that can be considered an HPt signature sequence. CsHPt1 contains this complete HPt signature sequence, which includes the 12 amino acid highly conserved motif for the HPt domain (Fig. 3). We hypothesize that CsHPt1 is a phosphorelay protein induced in early somatic embryogenesis in sweet orange as part of the signal transduction mechanisms involved in the expression of embryogenic potential.

Auxin, a phytohormone with diverse roles during plant development has been identified as a key signal molecule in the reactivation of the cell cycle and the initiation of embryo formation during somatic embryogenesis [44]. It is reasonable to infer that cytokinin signaling pathways are very active in early stages of plant embryogenesis as well. Cytokinins are essential plant hormones involved in numerous aspects of plant growth and development including cell division, shoot and root differentiation and photomorphogenic development [49]. All these processes are inherent to plant embryogenesis. Whether CsHPt1 is a phosphotransmitter protein involved in cytokinin signaling during early embryogenesis in sweet orange needs to be determined. It has been established that the perception and signal transduction of cytokinin signaling pathways involve a phosphorelay system [50]. Moreover, as previously mentioned, three of the five Arabidopsis phosphotransmitter AHP genes have been found to be part of cytokinin signaling pathways. HPt genes reported in other plant species including maize [40] and *Catharantus roseus* [41] are involved in cytokinin signaling as well. In a recent study on the endogenous hormone levels of embryogenic citrus callus cultures of different cultivars, high cytokinin levels were found in most of the cultures after glycerol induction [51], suggesting active cytokinin signaling pathways during early embryogenesis. It is possible that CsHPt1 encodes a protein that is part of such a signaling system mediating the embryogenic response in sweet orange. Alternatively, CsHPt1 may be involved in still unidentified signaling pathways involving His-containing phosphotransfer proteins not related to cytokinins.

Our real-time RT-PCR results showed that CsHPt1 transcripts in cell line V99 were more abundant in 35 days globular embryos than in embryogenic calli, confirming the results of our differential display experiments (Table 1). When CsHPt1 expression levels were tested in four other embryogenic cell lines, lines V89 and V98 showed similar results to line V99, with higher CsHPt1 transcript abundance in 35-day globular embryos than in embryogenic calli. In contrast, lines V97 and V00 showed no significant difference between the two embryogenic stages. It is possible that in these two cell lines expression of CsHPt1 increased transiently before or after the 35-days post-induction timepoint and went undetected in our study. Citrus embryogenic callus cultures have been found to differ in hormone levels depending on genotype and growing conditions [51]. It is not unreasonable to speculate that conditions such as endogenous hormone levels or small differences in environmental conditions during culturing may affect the timing of signaling factors that become active during the morphogenetic process taking place during early embryogenesis. Overall, or results indicate that CsHPt1 is induced in embryogenic calli, in globular-stage embryos (Table 1) and in tissues with embryogenic potential at higher levels than in non-embryogenic calli or in tissues without embryogenic potential (Fig. 5). This suggests a role for CsHPt1 as a phosphotransmitter intermediate during very early embryogenesis in *Citrus*. The fact that CsHPt1 is also expressed in other tissues tested, like phloem, flavedo and non-embryogenic calli (Fig. 5) means that this signaling factor is not specific to somatic embryogenesis but underlies other physiological processes unrelated to embryogenesis.

Single hybridization bands were observed in the genomic Southern blot of CsHPt1 except in lanes corresponding to *Eco*RI and *Xba*I (Fig. 6). The shorter bands generated by these two enzymes may be a result of multiple digestion sites and if pieced together will very likely resemble the size of the bands in all other lanes. This indicates CsHPt1 is present in one single copy in the citrus genome. These results are in contrast to those from other higher plant species where at least two HPt genes have been reported. The Arabidopsis genome, as mentioned earlier, contains at least five HPt genes [44], while maize [40] and *C. roseus* [52] contain three and two isogenes, respectively. Therefore, we searched the HarvEST v 0.34 citrus database (http://harvest.ucr.edu) for unigenes representing two-component phophorelay intermediates in *C. sinensis*. We found three other putative CsHPts (GenBank accession nos. CB292517, BQ624437, and CN181823). In all cases, their amino acid sequences contain the 32 amino acid HPt signature (data not shown); however, the rest of the sequences vary extensively between each other and compared to CsHPt1 (data not shown). Again, this was not surprising since amino acid sequences among HPt domains are poorly conserved [48]. Nucleotide sequence alignment between CsHPt1 and the three other CsHPts showed low homology as well (around 50% homology; data not shown). This explains why we observed hybridization signals in the Southern blot for CsHPt1 only. In the future, low stringency hybridization conditions may allow us to detect other HPts in the citrus genome.

Currently, only a few citrus species and cultivars respond to induction of somatic embryogenesis [51]. Because this process is critical in obtaining transgenic plants for genetic studies in this species, isolation of genes whose expression is altered upon early stages of somatic embryogenesis is a significant step towards understanding the difference in embryogenic potential of citrus cultivars.

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